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Kok, Jan; Klappe, Katharina; Hummel, Ina

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## Review Article

# The Role of the Actin Cytoskeleton and Lipid Rafts in the Localization and Function of the ABCC1 Transporter

**Jan Willem Kok, Karin Klappe, and Ina Hummel**

*Department of Cell Biology, University Medical Center Groningen, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands*

Correspondence should be addressed to Jan Willem Kok; [j.w.kok@umcg.nl](mailto:j.w.kok@umcg.nl)

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ATP-binding cassette (ABC) transporters are known to be important factors in multidrug resistance of tumor cells. Lipid rafts have been implicated in their localization in the plasma membrane, where they function as drug efflux pumps. This specific localization in rafts may support the activity of ABC/Abc transporters. This raises questions regarding the nature and composition of the lipid rafts that harbor ABC/Abc transporters and the dependence of ABC/Abc transporters—concerning their localization and activity—on lipid raft constituents. Here we review our work of the past 10 years aimed at evaluating whether ABC/Abc transporters are dependent on a particular membrane environment for their function. What is the nature of this membrane environment and which of the lipid raft constituents are important for this dependency? It turns out that cortical actin is of major importance for stabilizing the localization and function of the ABC/Abc transporter, provided it is localized in an actin-dependent subtype of lipid rafts, as is the case for human ABCC1/multidrug resistance-related protein 1 (MRP1) and rodent Abcc1/Mrp1 but not human ABCB1/P-glycoprotein (PGP). On the other hand, sphingolipids do not appear to be modulators of ABCC1/MRP1 (or Abcc1/Mrp1), even though they are coregulated during drug resistance development.

## 1. Introduction

The family of ATP-binding cassette (ABC/Abc) transporters is an important group of membrane-associated proteins that serve as transmembrane transporters for various substrates, including cytostatics employed to kill tumor cells. There is a body of evidence indicating that these transporters are associated with lipid rafts, as reviewed in a number of papers (e.g., [1–3]). In most studies lipid rafts were isolated biochemically, initially employing detergents and later also using detergent-free approaches. The membrane domains isolated using detergents were named detergent-resistant membranes (DRM) among other names. Likewise, in the case of detergent-free isolation we refer to these membrane domains as detergent-free membranes (DFM).

The association of ABC/Abc transporters with these membrane domains raises the question whether this has consequences for the activity of these proteins as drug efflux pumps. Indeed, the membrane environment may contribute to optimal activity of the transporter. Moreover, if there is

indeed a functional consequence of being in a lipid raft, it is challenging to investigate which components of lipid rafts may be instrumental as modulators of ABC activity. Such lipid raft components may function by keeping the ABC/Abc transporter associated with the lipid rafts and in addition they may modulate the protein by direct lipid-protein interactions. When considering these options, a few molecules immediately come to mind. Lipid rafts are known to be enriched in sphingolipids as well as cholesterol; hence it is conceivable that such lipids keep the ABC/Abc transporter localized to lipid rafts and thereby keep it active. In addition, in recent years it has become apparent that the actin cytoskeleton, more specifically cortical actin, has a role in stabilizing lipid rafts [2]. Therefore, the potential role of actin as a modulator of ABC localization and activity becomes an issue as well.

In this paper we review our work of the past 10 years which aimed to establish the type and composition of lipid rafts that harbor ABC/Abc transporters. Moreover, we systematically analyzed the contribution of each sphingolipid, cholesterol, and cortical actin to localization and activity

of ABC/Abc transporters. The focus was on the ABC/Abc transporter ABCC1/multidrug resistance-related protein 1 (MRP1) and its rodent counterpart Abcc1/Mrp1. We usually compared its behavior to that of the most studied ABC transporter ABCB1/P-glycoprotein (PGP). Our work provided several surprising results that together gave rise to a coherent picture with a major role for cortical actin in localization and function of ABC/Abc transporters. This review also discusses current models of membrane domain association of ABC/Abc transporters and various leads to future research aimed to resolve the issues that emanate from these models.

## 2. The Nature of the Lipid Rafts That Harbor ABC Transporters

It is known that there is not just a single type of lipid raft. For example, we distinguish caveolae and noncaveolar lipid rafts, the difference being the association of the protein caveolin-1 with lipid rafts of the type caveolae. Moreover, among the noncaveolar lipid rafts, it is considered that there is further heterogeneity [4]. This is based on the notion that, when using different detergents, one isolates membrane domains with different compositions in terms of lipids and proteins. One explanation is that different types of lipid rafts exist in cells, which differ in resistance towards solubilization by various detergents [4].

We investigated whether ABC transporters in tumor cells are localized in caveolae or in noncaveolar lipid rafts. In the human ovarian tumor cell line 2780AD it was evident that the overexpressed transporter ABCB1/PGP was not in caveolae. Caveolae were not present in these cells, as indicated by the absence of the protein caveolin-1 [5]. However, ABCB1/PGP did associate with DRM fractions that were isolated using the detergent Lubrol [5]. In the human colon tumor cell line HT29<sup>col</sup> caveolin-1 was present and these cells thus likely do express caveolae. However, the overexpressed transporter ABCC1/MRP1 in these cells did not colocalize with caveolin-1, as demonstrated by confocal microscopy. Furthermore, the use of Triton X-100 as detergent to isolate DRM resulted in separation of ABCC1/MRP1 and caveolin-1 in the DRM density gradient, indicating that the two proteins were associated with different types of rafts [5]. In conclusion, both ABCB1/PGP and ABCC1/MRP1 resided in noncaveolar Lubrol-based DRM but not in Triton X-100-based DRM [5].

In another study we carefully analyzed the composition of Lubrol versus Triton X-100 DRM and showed that Lubrol-based DRM contain more protein and lipid mass and are relatively enriched in aminophospholipids, while Triton X-100-based DRM are relatively enriched in sphingolipids [6]. Based on these studies we proposed a model for Lubrol-based DRM consisting of concentric layers, the core being equivalent to the Triton X-100-based DRM enriched in sphingolipids and the outer layer being enriched in ABC transporters and aminophospholipids, which are known to affect ABC function [7].

## 3. Glycolipids and ABC Transporter Localization/Function

Our early studies dating back to 2000 indicated a positive correlation between ABC expression and glycolipids, especially glucosylceramide. Firstly, ABCC1/MRP1 overexpressing HT29<sup>col</sup> cells showed increased levels of glucosylceramide [8], and this was also observed for ABCB1/PGP overexpressing 2780AD cells [9]. Moreover, there was a striking concomitance in the upregulation of both ABCC1/MRP1 and glucosylceramide during multidrug resistance acquisition in the transition from parental HT29 cells to resistant HT29<sup>col</sup> cells upon long treatment with colchicine [10]. Both the glycolipid and the transporter were enriched in Lubrol-based DRM [10]. Secondly, three different human neuroblastoma cell lines were selected with differential ABCC1/MRP1 and ABCB1/PGP expression and similar observations concerning a correlation with glucosylceramide levels were obtained [11]. In the neuroblastoma cell lines, also different ganglioside patterns were observed, opening the possibility to study potential relations between these complex glycolipids and ABC transporters [11]. Thirdly, stable transfection of HepG2 human hepatoma cells with cDNA coding for ABCB1/PGP resulted in a 3-fold increased level of lactosylceramide and an increased ganglioside mass. This was shown to be caused by transcriptional upregulation of the enzyme lactosylceramide synthase [12].

It should be noted that an increased pool of glucosylceramide may indicate activation of an alternative pathway in multidrug resistance which can function independent of ABC transporters. This pathway is an escape route from ceramide-induced apoptosis by conversion of ceramide into glucosylceramide through the action of glucosylceramide synthase [13]. Surprisingly, all our subsequent studies aimed at defining a role for glycolipids in modulation of ABC function or performing an ABC transporter-independent role in multidrug resistance failed to show such a correlation. In HT29<sup>col</sup> cells ABCC1/MRP1 was solely responsible for drug resistance. The ceramide level was strongly upregulated in multidrug resistant cells, arguing against an apoptotic role for this lipid [10]. Further enhancing the ceramide level by inhibition of glucosylceramide synthase using N-butyldeoxynojirimycin (NB-dNJ; Figure 1(a)) did not have an effect on resistance [10]. Moreover, in 2780AD cells, the increased glucosylceramide levels were not due to increased expression or activity of glucosylceramide synthase but rather to a block in lactosylceramide synthesis at the level of the Golgi apparatus [9].

Finally, we decided to rigorously establish potential effects of glycolipids by knock-down studies (Table 1). In human neuroblastoma cell lines the ganglioside content was reduced by 90% using the inhibitors D,L-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (t-PPPP) or NB-dNJ (Figure 1(a)). This had no effect on either ABCC1/MRP1- or ABCB1/PGP-mediated efflux [14]. Interestingly, the localization of ABCC1/MRP1 in DRM was not affected by t-PPPP, while it was shown that the GM1 level in DRM was more than 90% reduced [14]. Thus, these studies

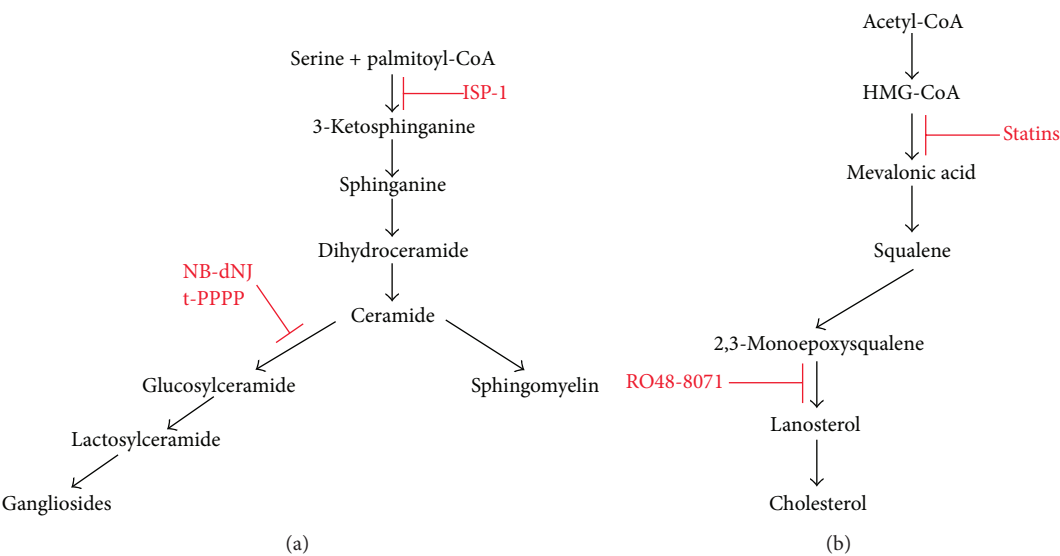


FIGURE 1: Metabolism of sphingolipids (a) and cholesterol (b) and the steps where the indicated inhibitors act. ISP-1 acts on the first step of sphingolipid metabolism, that is, the condensation of serine and palmitoyl-CoA. Both NB-dNJ and t-PPPP inhibit the conversion of ceramide into glucosylceramide by glucosylceramide synthase (a). Cholesterol biosynthesis is inhibited by statins, such as lovastatin, which act on an early step in the pathway by inhibiting HMG-CoA reductase. RO48-8071 inhibits a late step in the pathway, that is, formation of lanosterol by lanosterol synthase, which is an oxidosqualene cyclase enzyme (b).

TABLE 1: Dependence of ABC transporter localization (“Raft”) or function (“Efflux”) on lipids or cortical actin.

	ABCB1		ABCC1		Abcc1	
	Raft	Efflux	Raft	Efflux	Raft	Efflux
Glycolipid depletion	n.d.	= [14]	= [14]	= [14]	n.d.	n.d.
Sphingolipid depletion	n.d.	= [18]	= [17, 18]	= [17, 18]	= [17]	= [17]
Cholesterol depletion (CO)	n.d.	n.d.	= [21]	= [21]	= [21]	= [21]
Cholesterol depletion (CD)	n.d.	n.d.	= [21]	↓ [21]	↓ [21]	↓ [21]
Cholesterol depletion (Lo/Ro)	n.d.	n.d.	n.d.	n.d.	↓ [21]	= [21]
Cortical actin depletion	= [30, 31]	= [30]	↓ [24]	↓ [24]	↓ [24, 31]	↓ [24]

The table summarizes the effects of depletion of lipid (sub)families or cortical actin on ABC transporter localization in lipid rafts and its efflux function. This is indicated for the ABC transporters ABCC1 (human), Abcc1 (murine), and ABCB1. Numbers indicate literature references. CO: cholesterol oxidase; CD: methyl-β-cyclodextrin; LO/RO: lovastatin/RO48-8071; =: no effect; ↓: reduced lipid raft association or efflux function; n.d.: not determined.

led to the conclusion that although glycolipid levels change with upregulation of ABC transporters, these lipids are dispensable when it comes to ABC transporter activity (Table 1). Furthermore, the localization of ABC transporters in DRM is not dependent on gangliosides.

#### 4. Sphingolipids and ABC Transporter Localization/Function

At this point, it is important to note that with the mentioned inhibitors of sphingolipid metabolism one does not downregulate all sphingolipid classes as the block occurs

beyond the steps leading to ceramide synthesis (Figure 1(a)). Ceramide has been noted as an important constituent of DRM and together with sphingoid bases performs functions in cell signaling [15, 16]. Therefore it is of importance to devise a study aimed at downregulation of all sphingolipid classes, which we subsequently undertook using the inhibitor myriocin/ISP-1 (Figure 1(a)). Not all cell lines are equally susceptible to this inhibitor and some cell lines hardly respond in terms of downregulation of sphingolipids (Klappe, K., and Kok, J. W.; unpublished results). After ample screening, we found that murine Neuro-2a cells respond very well with around 90% depletion of the total sphingolipid pool after 3 days of treatment with myriocin/ISP-1. This cell line also

offers the advantage that it intrinsically expresses murine Abcc1/Mrp1, obviating the need for upregulation of the protein by gene transfection or selection with cytostatics. However, we did not want to rely solely on this cell line and also included BHK/MRP1 cells, a hamster cell line with forced expression of the human ABCC1/MRP1 gene. Inclusion of this model assured that results from these studies were not restricted to a single cell line and allowed comparison to our previous studies in human tumor cell lines expressing human ABCC1/MRP1. With these model cell lines, we were also able to compare the behavior of rodent Abcc1/Mrp1 and human ABCC1/MRP1.

In both Neuro-2a and BHK/MRP1 cells a very efficient downregulation of sphingolipids did not result in any effect on Abcc1/Mrp1 or ABC1/MRP1 efflux activity, respectively [17]. This shows that indeed all sphingolipid classes are dispensable with respect to ABC function (Table 1). An important aspect of this study was the assessment of lipid raft association of the ABC/Abc transporter. This was done elaborately using both detergent-based and detergent-free methods for isolation of lipid rafts and in all cases resulted in absence of any effect of sphingolipid inhibition on localization of the ABC/Abc transporter in lipid rafts [17]. Moreover, it was shown that in the lipid raft fractions sphingolipids were equally reduced compared to whole cells, a prerequisite for drawing any conclusions [17]. It was concluded that extensive sphingolipid depletion did not affect lipid raft localization and efflux function of the ABC/Abc transporter ABCC1/MRP1 (or Abcc1/Mrp1; Table 1).

## 5. Lipid Rafts and ABC Transporter Localization/Function

The inevitable conclusion emerged that extensive sphingolipid depletion did not affect lipid raft integrity per se. Indeed, myriocin/ISP-1 treatment did not affect the protein, glycerophospholipid, and cholesterol profile of the lipid raft isolation gradients [17]. Lipid raft markers such as Src, flotillin, and caveolin-1 showed similar distributions and extent of lipid raft association in myriocin/ISP-1 treated and control cells. Moreover, the cholesterol content was the same as well as the glycerophospholipid content and head group composition, both in whole cells and isolated lipid rafts. The only apparent difference in myriocin/ISP-1 treated cells was a skewing of the fatty acids of the residual sphingolipids towards  $C_{16:0}$  chains and a concomitant significant increase in phosphatidylcholine ( $C_{32:0}$ ) content, which likely represented two  $C_{16:0}$  chains [17]. This could be interpreted as a compensatory mechanism to support lipid raft maintenance.

Although sphingolipids apparently do not matter for ABC/Abc transporter localization and function (previous section), all results thus far mentioned still allow the conclusion that the localization of the ABC/Abc transporter in lipid rafts does matter for its activity. Indeed, subsequent studies showed a strong correlation between lipid raft localization on the one hand and ABC function on the other. In the context of myriocin/ISP-1 this became apparent when the incubation time with the inhibitor was extended

from 3 to 7 days. This regime did not further affect sphingolipid levels, which were already maximally reduced after 3 days of incubation. However, in contrast to the 3-day protocol, 7 days of treatment resulted in an increase in ABCC1/MRP1 efflux activity in BHK/MRP1 cells [18]. Concomitant with this increase, an increased association of ABCC1/MRP1 with lipid rafts was observed [18]. These studies were extended to measurements on ABC activity in plasma membrane vesicles isolated from myriocin/ISP-1 treated cells. Similar results, that is, increased activity of ABCC1/MRP1, were obtained [18]. This offered the possibility to perform the Michaelis-Menten analysis that revealed an unaltered intrinsic MRP1 activity ( $K_m$ ). However, the fraction of active transporter molecules ( $V_{max}$ ) increased, which was interpreted as an enhanced recruitment of MRP1 into lipid rafts, thereby promoting MRP1 activity [18].

## 6. Cholesterol Depletion Procedures

A potential role for cholesterol in modulation of ABC transporter activity has been well studied, especially regarding ABCB1/PGP [2, 3]. There are ample tools to deplete cholesterol in cells based on different molecular mechanisms. Firstly, physical extraction of cholesterol from the plasma membrane can be achieved by using cyclodextrins such as methyl- $\beta$ -cyclodextrin ( $M\beta CD$ ). Secondly, cholesterol oxidase (CO) treatment chemically converts cholesterol into cholestenone. Thirdly, statins such as lovastatin (LO) reduce cellular cholesterol by metabolic inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase (Figure 1(b)). Metabolic inhibition can be enhanced by combined treatment with lovastatin and RO48-8071 (RO), an inhibitor of oxidosqualene cyclase (Figure 1(b)). However, all of these procedures have their particular drawbacks;  $M\beta CD$  treatment has been shown to extract other molecules from membranes in addition to cholesterol, such as glycerophospholipids and even proteins [19, 20]. Statins not only inhibit the biosynthesis of cholesterol, but also that of isoprenoids such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate, which are synthesized from mevalonic acid (Figure 1(b)). Thus, statins can interfere with protein prenylation, which may well be relevant to lipid rafts. Cholesterol oxidase does not remove cholesterol but converts it to cholestenone, which may fulfill some of the properties of cholesterol, even though it lacks the hydroxyl group that is considered to be important for its biological functions. Taken together, we argue that it is not sufficient to perform a single procedure for cholesterol depletion, but instead 2 or 3 strategies should be used in parallel. Results should be consistent and not dependent on the method of choice.

## 7. Cholesterol and ABC Transporter Localization/Function

We have studied cholesterol dependence of ABCC1/MRP1 or Abcc1/Mrp1 localization and activity in Neuro-2a and BHK/MRP1 cells, respectively, using the three approaches for cholesterol depletion as mentioned in the previous section



([21]; Table 1). Cholesterol oxidase treatment did not affect ABCC1/MRP1- or Abcc1/Mrp1-mediated efflux, although the cholesterol level was strongly decreased, especially in cholesterol oxidase-treated BHK-MRP1 cells [21]. When Neuro-2a cells were treated with lovastatin plus RO48-8071 (RO/LO), there was no effect on Abcc1/Mrp1-mediated efflux. Moreover, with M $\beta$ CD treatment, efflux was dissociated from the cholesterol level, since short-term treatment did affect efflux in BHK-MRP1 but not Neuro-2a cells, whereas cholesterol levels were equally decreased in the two cell types [21]. Longer-term M $\beta$ CD treatment in the two cell lines decreased both cholesterol levels and ABCC1/MRP1- (or Abcc1/Mrp1-) mediated efflux. However, data obtained with MK571 as an inhibitor of ABCC1/MRP1- (or Abcc1/Mrp1-) mediated efflux showed that, after longer-term M $\beta$ CD treatment, substrate is lost from MK571-treated cells, suggesting that MK571 becomes toxic in combination with M $\beta$ CD [21]. This would indicate that membranes gradually destabilize with M $\beta$ CD treatment. The conclusion was that ABCC1/MRP1 (or Abcc1/Mrp1) activity was not correlated with cholesterol levels in these cells.

Similar results were obtained regarding the extent of lipid raft association of ABCC1/MRP1 (or Abcc1/Mrp1) in relation to cholesterol levels [21]. Thus, cholesterol is not essential for ABCC1/MRP1 (or Abcc1/Mrp1) function and does not seem to take part in the mechanism underlying functional association of ABCC1/MRP1 (or Abcc1/Mrp1) with lipid rafts (Table 1). Taken together with the data on sphingolipid modulation (previous paragraph), we came to the remarkable conclusion that two lipid classes—sphingolipids and cholesterol—which are enriched in lipid rafts and considered to be important for lipid raft function did not relate to ABC/Abc function in lipid rafts. The intriguing question thus came to mind how ABCC1/MRP1 (or Abcc1/Mrp1) is stabilized in lipid rafts and whether this localization is indeed relevant to function of the ABC/Abc transporter.

## 8. From Lateral to Transverse Interactions: Cortical Actin and ABCC1 Transporter Localization/Function

So far we have discussed potential interactions of ABC/Abc transporters with lipids in the lateral aspect of membrane domains. Now we shift gears to transverse interactions, since there is evidence that cortical actin plays a role in structuring lipid rafts. Studies with environment sensitive lipophilic fluorescent dyes have shown that lipids in actin-rich membrane domains are condensed relative to remaining regions of the plasma membrane, which indicates that they occur in a relatively ordered state [22]. Treatment with latrunculin B disrupted the actin cytoskeleton and resulted in decondensation of the plasma membrane ([22] and references therein). Using fluorescence resonance energy transfer (FRET) analysis, latrunculin B was shown to disrupt coclustering of lipid raft-associated proteins, probed with fluorescent tags [23].

We investigated whether ABCC1/MRP1 (or Abcc1/Mrp1) localization in lipid rafts and its functional activity as an efflux pump were affected by disruption of the cortical actin cytoskeleton ([24]; Table 1). Firstly, it was important to be able to attribute potential effects specifically to the cortical actin, as opposed to actin stress fibers. To do so, we tested several actin disrupting agents in various concentrations and found conditions where only stress fibers were disrupted, but not cortical actin, using cytochalasin D. In contrast, latrunculin B in the used concentration disrupted both cortical actin and stress fibers [24]. This allowed us to ascribe effects occurring with latrunculin B, but not cytochalasin D, to cortical actin involvement. Latrunculin B treatment, but not cytochalasin D treatment, resulted in loss of lipid raft association of Abcc1/Mrp1 (Neuro-2a cells) and ABCC1/MRP1 (BHK/MRP1 cells). Concomitantly, the efflux activity of the transporter was reduced under conditions of latrunculin B only and in both cell lines [24]. The effect was reversible when the cortical actin cytoskeleton was allowed to recover after withdrawal of latrunculin B. Moreover, an opposite effect on ABCC1/MRP1 activity was observed when the actin cytoskeleton was stabilized using jasplakinolide [24]. These results strongly suggested a correlation between the localization of the ABC/Abc transporter in lipid rafts and its efflux activity, both being dependent on intact cortical actin.

However, to firmly establish this conclusion, one hurdle needed to be overcome, as it was noted that latrunculin B treatment also resulted in increased intracellular staining of ABCC1/MRP1 (or Abcc1/Mrp1). This was shown to be due to increased endocytosis of the ABC/Abc transporter under conditions of cortical actin disruption and such an effect could potentially contribute to a reduction of efflux activity occurring at the plasma membrane [24]. Apparently, cortical actin acted as a fence reducing endocytosis in control cells. When latrunculin B treated cells were preincubated with nocodazole, which resulted in disruption of microtubules, the effects of latrunculin B were counteracted. This occurred for all effects of latrunculin B, that is, lipid raft association, efflux function, and endocytosis of the ABC/Abc transporter [24]. Indeed, it was shown that nocodazole pretreatment precluded cortical actin disruption by latrunculin B, suggesting a role for microtubules in effective cortical actin breakdown. However, this did not help with deciphering whether the reduced efflux activity of ABCC1/MRP1 (and Abcc1/Mrp1) under conditions of cortical actin disruption was related to a reduced lipid raft association of the transporter or due to its endocytic uptake.

Fortunately, the use of an inhibitor of endocytosis, tyrphostin A23, provided the answer. Preincubation with tyrphostin A23 abrogated endocytic uptake of the Abc transporter but did not counteract the effects of latrunculin B on both efflux activity and extent of lipid raft association of Abcc1/Mrp1 [24]. In conclusion, strong evidence was obtained for a role of the cortical actin cytoskeleton in stabilizing the lipid raft association of ABCC1/MRP1 (and Abcc1/Mrp1) and hence supporting its efflux activity (Table 1). This study further emphasized a correlation

between lipid raft localization and function of the ABC/Abc transporter.

## 9. Cortical Actin and ABCB1 Transporter Localization/Function

We next wondered if this also occurred for other ABC transporters. In the case of ABCC2/MRP2 there is ample evidence that actin plays a role in its localization and function. However, in this case a molecular link to actin has been discovered, since ABCC2/MRP2 is hooked up to actin via ezrin/radixin/moesin (ERM) proteins [25, 26]. It is not likely that such a direct link occurs in the case of ABCC1/MRP1 as this protein lacks actin-binding motifs found in ABCC2/MRP2. Apart from the ABCC/MRP subfamily, ABCB1/PGP is interesting to study in this respect. Several reports indicated a link between ABCB1/PGP and actin [27], also involving the participation of ERM proteins [28, 29]. In view of our results on ABCC1/MRP1 (or Abcc1/Mrp1) in relation to cortical actin, we therefore tested whether ABCB1/PGP was localized in actin-dependent lipid rafts and depended on intact cortical actin for its efflux function. We investigated this in two cell lines, including the NIH 3T3 mouse fibroblast cell line transfected with human MDRI (NIH 3T3 MDRI G185). In addition, a cell line with endogenous expression of ABCB1/PGP was chosen, the SK-N-FI human neuroblastoma cell line [30].

In both cell lines, latrunculin B did not affect ABCB1/PGP-mediated efflux (Table 1). This was rigorously tested using two different assays (rhodamine 123 and calcein-AM), both with three different substrate concentrations [30]. Latrunculin B was shown to have no effect on the surface expression of ABCB1/PGP, in spite of severe changes in cell and membrane morphology. In NIH 3T3 MDRI G185 cells ABCB1/PGP was mostly localized in nonlipid raft membrane areas. ABCB1/PGP associated with lipid rafts was mostly found in gradient fractions 3-4 instead of 1-2 (see below), and the amount found in fractions 3-4 did not change with latrunculin B treatment [30]. In conclusion, ABCB1/PGP in two cell lines was not affected by cortical actin disruption concerning both its efflux function and its membrane localization (Table 1). The total membrane pool remained similar as well as the distribution between lipid raft and nonraft membrane areas. ABCB1/PGP thus behaved very differently from ABCC1/MRP1.

## 10. Separation of Actin-Dependent and Actin-Independent Lipid Rafts

In the course of our studies, we observed that the gradient fractions 1-2 versus 3-4 from the detergent-free lipid raft isolation procedure were differently affected by latrunculin B treatment. We decided to systematically investigate this and obtained data supporting the conclusion that both the pools 1-2 and 3-4 of the gradient fractions contain lipid rafts/DFM [31]. Indeed, both pools fulfilled the criteria for lipid rafts as they contained floating material and displayed enrichment of both cholesterol and sphingolipids relative

to protein content, when compared to nonraft fractions 5-6. Both pools contained only very small amounts of the nonraft marker Rho-GDI. Upon latrunculin B treatment, cholesterol and sphingolipids partly shifted from fractions 1-2 to 3-4. Moreover, Abcc1/Mrp1, actin, and Src partly shifted out of fractions 1-2 but not out of fractions 3-4 [31]. We concluded that we had obtained a method to separate actin-dependent lipid rafts/DFM (fractions 1-2) from actin-independent lipid rafts/DFM (fractions 3-4) and obviously also from nonraft membranes (fractions 5-6).

## 11. Conclusion

The overall conclusion is that ABCC1/MRP1 (or Abcc1/Mrp1) is enriched in lipid rafts in all the cell types we have studied. Human ABCC1/MRP1 and rodent Abcc1/Mrp1 behaved similarly in all aspects of our studies. These lipid rafts are characterized by a high lipid/protein ratio; in other words, the ABC/Abc transporter is found in membrane domains that are relatively poor in proteins. Moreover, ABCC1/MRP1 (or Abcc1/Mrp1) is mostly found in actin-dependent lipid rafts. This localization confers a functional dependence on cortical actin of the transporter in terms of its efflux activity. Cortical actin is localized just beneath the plasma membrane and stabilizes lipid rafts and therefore also ABCC1/MRP1 (or Abcc1/Mrp1) which is localized in these rafts. The transporter is stabilized concerning residence in the plasma membrane, localization in a lipid raft, and functional activity. In contrast, ABCB1/PGP appears to be insensitive to cortical actin disruption, at least in the cell types we studied. It should be noted that other labs did find a link between this transporter and the actin cytoskeleton and this may depend on the cell type in question [27-29].

### 11.1. Future Perspectives: ABC Transporters in Lipid Rafts.

There are some important issues that remain to be explored, with respect to both ABC/Abc transporter localization/function and membrane structure in a more general sense. The latter refers to the notion that our work and that of many other labs may shed new light on how membranes are organized, specifically concerning the importance of the cortical actin cytoskeleton, as well as the interplay between cortical actin and lipid rafts. Firstly, it is highly intriguing how ABCC1/MRP1 (or Abcc1/Mrp1) is stabilized by actin in lipid rafts, given that it is not linked to actin in a molecular sense, bridged by ERM proteins, as is the case for ABCC2/MRP2. In a previous review we have proposed a model indicating 5 ways in which an ABC/Abc transporter could be embedded in the membrane in relation to actin and/or lipid rafts [2]. Concerning ABCC1/MRP1 (or Abcc1/Mrp1) we favour the option that it is localized in lipid rafts, which also contain a hypothetical raft actin linker protein (RAL) with which the ABC/Abc transporter may interact (Figure 2(a)). This RAL will then assure that also ABCC1/MRP1 (or Abcc1/Mrp1) is restricted in lateral movement by actin. Clearly, this model needs supportive evidence and we think it is worthwhile to identify potential

interaction partners for ABCC1/MRP1 (or Abcc1/Mrp1) in lipid rafts.

On the other hand, there is an alternative explanation that obviates the need for lipid raft involvement. This is based on the anchored protein picket (APP) model [32, 33], in which transmembrane proteins anchored to cortical actin just underneath the plasma membrane divide the membrane in a number of corrals. Proteins and lipids in a certain corral can move freely inside that corral but are restricted in movement across the picket fence to the next corral. Applied to ABCC1/MRP1 (or Abcc1/Mrp1), this would mean that the transporter is temporarily confined to a specific environment where it can function optimally by the transmembrane protein picket fence surrounding its corral (Figure 2(b)). A simplified version of this model is the fence model [32], stating that transmembrane proteins, such as ABCC1/MRP1 (or Abcc1/Mrp1), are temporarily confined to a corral because their cytoplasmic domains collide with the cortical actin cytoskeleton just underneath the plasma membrane (Figure 2(c)). In our view, much is to be expected from the emerging interactions of ABC/Abc transporters with the actin cytoskeleton in a lipid raft context. Indeed, manipulation of actin may be a good tool to interfere with lipid raft integrity and look for effects on ABC/Abc transporter localization and function without changing the cellular lipid composition. Moreover, the intriguing hypothesis that ABC/Abc transporter function is regulated by a two-axis system, combining lateral interactions with lipids and/or proteins in the plane of the membrane with transverse interactions with the actin cytoskeleton (and possibly integrins linking to the extracellular matrix (ECM) on the opposite side of the plasma membrane) requires attention. We anticipate that, in analogy to the developments in the fields of B cell receptor (BCR) and T cell receptor (TCR) signalling, lipid raft-actin cytoskeleton interactions will become an important theme in ABC/Abc transporter cell biology [2].

**11.2. Future Perspectives: Lipid Rafts and the Actin Cytoskeleton.** Zooming out from the focus on ABC/Abc transporters another issue becomes prominent, which is that of membrane organization in general. Since the fluid mosaic model was proposed in 1972 by Singer and Nicolson [34], several adaptations of this model have been suggested. These adaptations have been nicely summarized and discussed by Goni [35]. One important adaptation deals with lateral segregation of proteins and lipids and formation of membrane domains. We have already discussed the fence and anchored protein picket models, which involve the existence of corrals in which molecules can be temporarily trapped and in which the cortical actin cytoskeleton plays a prominent role in defining the borders of the corrals [32, 33]. Then there is the lipid raft hypothesis, proposed by Simons and Ikonen in 1997, involving membrane domain formation based on segregation of sphingolipids and cholesterol [36]. It is a major challenge to reconcile “raft” theory with “fence” theory, as exemplified in the case of ABC transporters discussed in the previous section. Are these theories exclusive or compatible with each other? Owen and Gaus discuss the possibility that

the cytoskeleton actually causes an increase in membrane lipid order, potentially involving local nucleation sites (“pinning”) for the development of ordered-phase domains [37]. Ehrig et al. presented a minimum realistic model for membrane rafts based on Monte Carlo simulations of two-component lipid membranes showing that phase separation can be strongly affected by interaction with the cytoskeleton [38]. A study by Lillemeier et al. [39] based on transmission electron microscopy of plasma membrane sheets showed that most or all plasma membrane-associated proteins are clustered in cholesterol-enriched domains that are separated by protein-free and cholesterol-low membrane. The authors propose the “protein island” model, where all membrane-associated proteins are clustered in protein islands, which can be subdivided into raft and nonraft islands. The protein islands are restricted in their lateral movement in the plane of the membrane due to connection to the actin cytoskeleton, which plays an important role in their formation and/or maintenance [39].

In addition to the cytoskeleton on the cytoplasmic side of the plasma membrane, one can also envision membrane organizing effects of exoplasmic molecules. The extracellular matrix may well have lipid raft modulating capacity, although such effects have not been modelled so far. In this respect, exogenous molecules such as DNA when they interact with the plasma membrane could also exert strong effects on membrane organization including lipid demixing. DNA interactions with cationic lipid membranes have been studied extensively and recently modelled [40] and this provides insights into the capacity of DNA to recruit lipids, induce domains, and deform membranes, although it should be realized that plasma membranes are not cationic membranes.

**11.3. Future Perspectives: Lipid Raft Modeling.** The lipid raft hypothesis has been subject to discussion and criticism up to the point where the mere existence of lipid rafts in living cells was coined as one of the five cell mysteries that need to be clarified [41]. Klotzsch and Schutz have recently published an elegant review in which they discuss the pros and concerns of different technological approaches to study membrane domains in the context of 10 propositions [42]. They also state their own thoughts about the consequences of the propositions and express a belief in lipid immobilization, phase separation in natural membranes, and the membrane skeleton acting as a template for protein and lipid organization [42].

It is important to keep refining the existing methodology for lipid raft isolation and to develop new technology to further advance our knowledge on this intriguing concept. In this respect, several developments have and will continue to increase our knowledge and understanding of membrane domains. First, development of novel superresolution microscopy techniques, for example, photoactivated localization microscopy (PALM), stimulated emission depletion (STED), and three-dimensional structured illumination microscopy (3D-SIM), has been crucially important in validating the concept of lipid rafts after the era of detergent solubilization of membranes [37, 43]. These techniques have



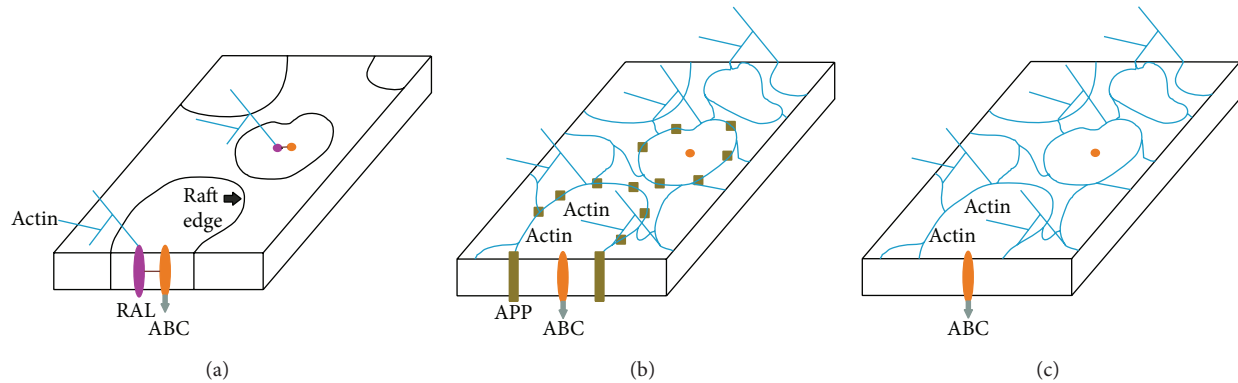


FIGURE 2: Models of ABC transporter localization in membrane domains and its interactions with the cortical actin cytoskeleton. The plasma membrane is viewed from the inside of the cell showing the inner face of the membrane. Three models are depicted. (a) The lipid raft model of ABC transporter retention in a domain. The ABC transporter is restricted in lateral movement by virtue of being in a lipid raft. The lipid raft is stabilized by the cortical actin cytoskeleton through the hypothetical protein RAL, which is linked to actin. The ABC transporter in turn may interact with RAL. (b) The anchored protein picket model of ABC transporter retention in a domain (adapted from [32]). The ABC transporter is restricted in lateral movement by virtue of colliding with picket proteins that are linked to cortical actin and fence the corral in which the ABC transporter is temporarily contained. (c) The fence model of ABC transporter retention in a domain (adapted from [32]). The ABC transporter is restricted in lateral movement by virtue of the collision of its cytoplasmic domain with cortical actin just underneath the plasma membrane, which forms a fence surrounding the corral in which the ABC transporter is temporarily contained. No distinction is made between human ABC and rodent Abc transporters for reasons of simplicity. RAL: raft actin linker protein; APP: anchored protein picket.

generally led to the conclusion that lipid rafts are rather small (down to 20 nm) and highly transient. However, in the field of plant and fungi cell biology, it has been recognized that membrane domains can be stable entities as well, while there are various principles by which they can be organized in addition to lateral separation of membrane lipids, as in lipid rafts [43]. Second, going hand in hand with development of sophisticated microscopy techniques is the use and development of fluorescent probes to label and investigate the properties of membrane domains. Klymchenko and Kreder have elegantly reviewed the available probes, which can be divided into the class of probes that preferentially partition into liquid ordered or liquid disordered phases and the class that displays phase dependent color, intensity, or life-time properties [44]. They argue that there is an urgent need to develop improved probes, especially for studies in living cell plasma membranes. Thirdly, system approaches are coming of age to unravel the complex cell biological consequences following an initiating event in membrane domains, for example, in cell signaling. The combination of network analysis, using either protein-protein interaction networks or mixed molecular networks, and quantitative proteomics with temporal or spatial resolution offers great potential for mapping of downstream pathways [45]. Fourthly, lipid rafts have entered the realm of soft matter physics, where theoretical models and computer simulations can lead to understanding of fundamental properties of membrane domains. Important questions, such as what the boundaries of membrane domains look like and whether the two membrane layers of lipid rafts are coupled or behave independently in terms of phase separations, are approached with this methodology [46, 47]. In model combined monolayer membranes, a phase separated monolayer can indeed induce phase separation in

the other monolayer, which originally was homogeneous. Line active molecules or “linactants” (2-D analogs of surfactants) are able to reduce the line tension that naturally occurs between domains. Thus, line active molecules allow membranes to adopt phase separated finite size domains that are stable despite a large interfacial length. Hybrid lipids with one saturated and one unsaturated fatty acid can perform the function of linactant in cellular membranes [46, 47]. In single particle tracking (SPT) analysis data are analyzed either at the single-molecule level or by pooling the data. The mean square displacement (MSD) is a measure of the average distance a molecule travels and is defined as the square of the distance a molecule travels over time interval  $t$ , averaged over many such time intervals (time average). Often this quantity is averaged also over all molecules in the system (ensemble average). When the MSD is plotted against time, motion can be classified according to the shape of the curve as (1) normal (Brownian) diffusion, (2) anomalous diffusion, (3) corralled/confined motion or (4) directed motion (flow) + diffusion [48]. Anomalous (non-Brownian) diffusion is often observed in cell membranes using SPT and interpreted to be the result of obstacles to diffusion or traps. Confined motion may result from corrals formed by the cytoskeleton, tethering to immobile proteins or from restrictions to motions imposed by lipid domains. Directed motion involves active transport executed by molecular motors along the cytoskeleton [48]. Cherstvy et al. [49] have recently published a computer simulation study on the physical properties of 2-D diffusive motion of large particles such as viruses or beads in mammalian cells. In their model they considered passive, thermally driven diffusion of small viruses in a model cell. The cell displays variation in local diffusivity in the cytoplasm due to heterogeneity of the density of macromolecular

crowding, the cytoskeletal meshwork, and the cellular organelles. Such forms of crowding impair the virus diffusivity inside the cell and may alter the motion from the Brownian to anomalous diffusion. They show that the heterogeneous diffusion process is weakly nonergodic because the time and ensemble averages of the MSD behave differently, which has important consequences for the proper physical interpretation of single particle tracking data. Moreover, the diffusion in the direction of the diffusivity gradient (radial) was shown to be anomalous, while the azimuthal diffusion was Brownian [49]. This study is relevant to the lipid membrane as well, which is now known to be crowded with proteins and likely in a heterogeneous fashion. 2-D diffusive motion of lipids and proteins in the lipid membrane may thus be described as a heterogeneous diffusion process due to heterogeneity of the density of integral membrane proteins and cytoskeletal and ECM proteins attached to either side of the bilayer. This may lead to anomalous diffusion, which is a property of lipid rafts. Protein crowding is indeed another important adaptation of the fluid mosaic model as proposed by Goni [35].

Based on our studies with myriocin/ISP-1 we would like to emphasize that the exact role of sphingolipids in forming and stabilizing lipid rafts needs to be more extensively studied and documented, preferably in various cell models. Our results discussed above raise doubts as to whether sphingolipids are essential components of lipid rafts. In a recent study a novel technique of secondary ion mass spectrometry to visualize isotopically labeled sphingolipids within the plasma membrane was used. Sphingolipid assemblages were identified as not being lipid rafts, as they are not dependent on cholesterol depletion. Instead, they are a distinctly different type of sphingolipid-enriched plasma membrane domain that depends upon cortical actin. Cholesterol was uniformly distributed throughout the plasma membrane and was not enriched within sphingolipid domains [50, 51].

Taken together, we can safely say that the future holds great promise for obtaining new insights into and building new models on the organization of the plasma membrane, a topic of fundamental importance. We believe that a precise understanding of the organizing role of the cortical actin cytoskeleton in membrane structure in general and lipid rafts in particular is of utmost importance and will be pursued actively in the near future. This may ultimately lead to a new and widely accepted model of membrane organization, which takes into account both lateral (lipid phase separation, protein crowding) and transverse organization principles (cytoskeleton; ECM) that together sculpt the membrane in a temporal fashion. Such a model will thus lift the lipid membrane from a 2-D to a 4-D structure.

## Abbreviations

ABC/Abc:	ATP-binding cassette
APP:	Anchored protein picket
BCR:	B cell receptor
CO:	Cholesterol oxidase
DFM:	Detergent-free membrane
DRM:	Detergent-resistant membrane

3D-SIM:	Three-dimensional structured illumination microscopy
ECM:	Extracellular matrix
ERM:	Ezrin/radixin/moesin
FRET:	Fluorescence resonance energy transfer
LO:	Lovastatin
M $\beta$ CD:	Methyl- $\beta$ -cyclodextrin
MRP1/Mrp1:	Multidrug resistance-related protein 1
MSD:	Mean square displacement
NB-dNJ:	N-Butyldeoxynojirimycin
PALM:	Photoactivated localization microscopy
PGP:	P-Glycoprotein
t-PPPP:	D,L- <i>threo</i> -1-Phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol
RAL:	Raft actin linker protein
RO:	RO48-8071
SPT:	Single particle tracking
STED:	Stimulated emission depletion
TCR:	T cell receptor.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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